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Research Article

Histological Characterization of Modified Calcite Paste as Pulpotomy Material in Partially Pulpotomized Rabbit Incisors

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ABSTRACT

Background: increasing the demand for vital pulp therapy (VPT) as a minimally invasive treatment, evolving the need for bio-inspired capping material to optimize the outcome with more predictable results. Aim: is to evaluate the biological efficacy of modified calcite paste as potential material for pulpotomy in terms of pulpal inflammatory response, dentin bridge formation and morphology. Methods: 24 lower central incisors of New Zealand rabbits were used, subdivided into two groups of 12 teeth according to sacrificing time (1 and 4 weeks), in each group, six teeth were used as the control group, the pulp is traumatically exposed and partially amputated left free of capping material, and six teeth used as the experimental group, were the amputated pulp capped with Modified Calcite (MC) paste, the cavity of both groups sealed with resin modified glass ionomere cement (pulpdent, USA).animal were sacrificed and teeth were collected for histological examination. Results: At 1-week and 4-week periods respectively, both groups showed non-significant differences in inflammatory extent, a highly significant difference in calcific bridge formation (P=0.002) and dentin morphology (P=0.002). The MC group showed faster dentin bridge formation with favourable morphology according to the scoring system in both periods compared to the control group. Conclusions: MC composite is a promising novel pulpotomy material.
Keywords: Pulpotomy, Calcium carbonate, Calcite, Rabbit incisor.

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INTRODUCTION

Pulpotomy is one of the vital pulp therapy (VTP) modalities, involving the surgical removal of a small portion (partial pulpotomy) or all the inflamed coronal pulp (full pulpotomy) and applying of the biocompatible material directly on the remaining pulp tissue, as a means to preserve the vitality of the remaining radicular pulp tissue, Pulpotomy procedure considered important procedure in pedodontics field, and the most common example would be the maintaining of radicular pulp tissue for apexogenesis in a recently erupted permanent tooth.[1] Recently, the application of pulpotomy has been extrapolated, to be a viable treatment option, an alternative to root canal therapy (RCT) in mature teeth even with irreversibly inflamed pulps.[2] Ultimate treatment success is highly dependent on the type of capping material.[3] More than 20 types of biomaterials have been advocated as pulp capping material with varying degrees of clinical success, but till now, an ideal capping material has not been established yet.[4,5]

Calcite is the most stable crystalline form of $CaCO_3$ polymorphs, nowadays, calcite is a ceramic material of high scientific interest as it is a naturally occurring cheap inorganic biomaterial, originated from varieties of daily renewed resources like eggshell, limestone, coral, coccolithophores, plant ashes, chalk and marble which is recently being studied in the biomedical field due to its excellence biocompatibility, porosity and pH sensitivity.[6,7] In many experimental animal studies, calcite derived from eggshells has been described to be an effective osteoconductive and biocompatible biomaterial.[8,9] In the dental field, calcite has been added to pulp capping material like MicroMega MTA, biodentine, and ProRoot MTA, and according to the manufacturers, it's used as a filler to enhance the mechanical properties and hydration reaction.[10-13] Furthermore, the biological performance of these capping materials seems to be greatly enhanced by the addition of $CaCO_3$in comparison to other capping materials of low content like pure MTA and calcium hydroxide,[14] this evidence suggests that the calcite is biocompatible and osteoconductive and may be suitable to be used as pulp capping material.

Pure calcite is relatively inert in simulated body fluid (SBF), it poorly induces calcium phosphate formation, as the apatite formation is directly related to calcium release in SBF.[15] Moreover, this crystalline form of $CaCO_3$ polymorphs is inert in vivo.[16,17] In the present study, by mixing calcite with diluted acetic acid we intended to enhance Ca ion bio-availability in the slightly alkaline environment as well as the surface porosity of calcite cement. Consequently, the bioactivity of calcite is a potential capping material, however, by trial, simple incremental mixing of calcite powder with diluted 0.5% acetic acid, is done to achieve paste with the best handling properties. However, such a reaction is a dissolution–precipitation reaction that can produce calcium acetate in addition to precipitated unreacted calcite as filler according to the following reaction in the equation of equilibrium reaction.[18,19]

$$(CaCO_3 + 2CH_3COOH \leftrightarrow Ca(CH_3COO)_2 + H_2O + CO_2) \quad (1)$$

$$(CaCO_3 + 2CO_2 + H_2O \leftrightarrow Ca^{+2} + HCO_3) \quad (2)$$

Acetic acid consumption and evaporation of CO_2 lead to cessation of the reaction.[20] The end product will form a reactant $CaCO_3$ embedded in a supersaturated solution of calcium acetate. During

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the initial setting the CO_2 bubbling leads to enhanced porosity and pore size leading to the forming of larger pores and channels of the paste,[21] which enhance the surface characteristic of calcite favorable for cell attachment and mineral deposition[22]. Moreover, both the acetate conjugate base (CH3COO−) from calcium acetate and carbonate(CO_3^{2-}) from calcite increase the effluent alkalinity of acetic solution from acidic pH to slightly alkaline Ph.[23] Furthermore, invitro has been found the hydrolysis of CH3COO−will potentiate apatite formation in the following reactions.[24]

$CH_3COO- + H_2O \rightarrow CH_3COOH + OH-$ (3)

$10Ca_2+ + 6PO_3- 4 + 2OH- \rightarrow Ca_{10}(PO4)_6 (OH)_2$ (4)

Based on all this in vitro evidence, the mixture of calcium carbonate and calcium acetate may be practicable to develop novel materials with potential use as pulpotomy paste in VPT.

MATERIALS AND METHODS

Materials: Materials used are precipitated extra pure calcium carbonate $CaCO_3$ (India, Mumbai). Glacial acetic acid was supplied from Merck, Germany. Light cure resin-modified glass ionomer cement (Prime Dent, USA)

MC paste preparation: 0.5% of diluted Glacial acetic acid is prepared, by adding 0.5 ml of Glacial acetic acid to 99.5 ml of distilled water. By in situ preparation the paste was prepared by mixing powder phase $CaCO_3$ with the liquid phase of 0.5% Acetic acid, the powder to liquid ratio chosen after a pilot study revealed that it had better handling properties to be used as pulpotomy paste[25,26,27] 3 Drops of diluted glacial acetic acid (0.5%) was mixed with 1 full spoon of calcium carbonate powder each full spoon weight 25 mg of pure calcium carbonate, mixing incrementally for two minutes then apply into Sterile disposable syringe (1ml) to facilitate its delivery to dress the exposed pulp.

Sample size and animal grouping: this research is done according to ethical approval from the research ethics committee of the College of Dentistry, University of Baghdad (Ref.no:462). According to previous studies, a minimal sample size was used.[28,29] The two lower permanent central incisors of Twelve healthy New Zealand male rabbits weighing 2.5 Kg were used in this study and randomly grouped as:

Group I (Positive control group, n=12 teeth): the pulp is mechanically exposed and partially pulpotomized without any capping material

Group II (Experimental group, n=12 teeth): the pulp is mechanically exposed and partially pulpotomized then capped with the experimental MC paste

The experimental teeth were obtained at interval periods (1,4 week, six teeth of each group per interval) and were collected and prepared for routine histological examination.[28,29]

Pulp capping procedure: The surgical procedure was done under aseptic conditions and a gentle operative technique. the dose of general anesthesia calculated according to each animal weight, using of Intra muscular injection of xylazine HCl 2% (5 mg/kg), followed by another of Ketamine HCL(35 mg/kg) to induce general anesthesia, after achieving good sedation, Class V cavities were prepared in the cervical buccal surface 1 mm incisal to the marginal gingiva by using of a high-speed round diamond 0.8 mm carbide tungsten bur (Komet Dental, Lemgo, Germany) with intermittent drilling under irrigation with sterile normal saline, drilling done at a 45 degrees inclination apically and extended for the pulp chamber, the pulp exposure done by using small spoon excavator, removing part of pulp tissue , the cavity formed is approximately of 2 mm depth necessary to apply capping material with final restoration,[30] the bleeding at the exposure site was controlled by irrigation with 5.25% concentration of NaOCl for 30 Sc then the cavity washed with distilled water and dry by brief air blot, the cavity of Group I left empty and just covered with resin modified glass ionomere cement were cured for 30 Second and the cavity of Group II filled with paste-like mixture of the novel cement then covered with resin modified glass ionomere cement were cured for 30 Second.[30,31]

Histologic Assessment: Six rabbits were euthanized per interval by intravenous injection with anaesthetic overdose. Jaws were fixed in 10% neutral buffered formalin, decalcified in 10% nitric acid for 3 days, dehydrated sequentially in graded alcohols, 70%, 80%, 90%, and 100%, then immersed in xylene, and finally embedded in paraffin blocks. The blocks were serially catted with an average thickness of 5 mm along the labiolingual direction passing through the centre of the pulp exposure site. The sections were stained with hematoxylin and eosin for histologic observations under light microscopy.[32] Each specimen was given a code to hide the specimen's identity (i.e., the material used and time interval) and avoid a possible bias. All specimens were included in the analysis, and none were excluded. Representative sections were examined and evaluated histologically, using light microscopy (Leica microscope) which includes a digital camera mounted on it. The extent of the inflammatory reaction was evaluated using the scoring system used by previous study[31] and continuity as well as the morphology of the dentinal bridge was evaluated according to a modified scoring system used by previous studies (Table 1).[33,34]

Histological analysis was done blindly by two evaluators under a light microscope, the reliability of the assessment was determined by kappa analysis, which resulted in k values above (80%) which represents a good value.[35]

Statistical Analysis: The detailed description for each variable was made on the Statistical Package for Social Sciences (SPSS) version 26. Fisher's exact test was used to assess the relationship between categorical variables. A confidence level of 95% with p value equal to or less than 0.05 was considered significant.

RESULTS

First-week histological analysis:

Results of the samples from the control group revealed that 83.3% moderate inflammatory response corresponding to score two where the inflammatory cell infiltration and dilated blood vessel extending to the middle part of the pulp, 16.7% mild inflammatory response, the necrotic tissue was observed at the exposure site. The pulp tissue subjacent to the necrotic zone shows a loss of the odontoblast layer with tissue disorganization, exhibiting tissue fibrosis with new blood vessel formation and inflammatory cell infiltration. However, the middle part of the pulp shows dilated and congested blood vessels while the odontoblast cells at the periphery of the pulp are arranged in palisade form. Regarding the formation of dentin bridge, all cases show deposition of new discrete calcified tissue on the wall of the pulp cavity beneath the exposure site, this calcified tissue of a tubular pattern which corresponds to score three (Table 2, Figures 1A, B, C, and D).

MC group shows 83.3% of samples show mild inflammatory response where the inflammatory cell infiltration seems to be within the most coronal part of the pulp adjacent to capping material, 16.7% of sample shows moderate inflammatory response, under the exposure site all cases show losing of odontoblast layer being replaced by partially mineralize thin incomplete dentin bridge and dense fibrous tissue with cell inclusion, the middle part of the pulp shows few dilated blood vessel, and well tissue organization, the odontoblast cell arrange in palisade form regarding dentin bridge formation all cases show partially formed dentine bridge with the mixed architecture of tubular and tubular dentin corresponding to score two for dentin bridge formation and bridge morphology (Table 1, Figures 1 E, F, G, and H).

Table 1: Scoring system for histological evaluation.

Scores	1	2	3	4
Inflammatory extent	Mild response subjacent to exposure site	Moderate: inflammatory cells are observed in the part of the coronal pulp	Severe: all coronal pulp up to apical region	Complete necrosis
Hard tissue continuity	hard tissue deposition as complete and continuous dentin bridge	Incomplete and discontinuous	a layer of scattered and foggy hard tissue deposition on the wall	Absences
Quality of dentin formation in the bridge	Regular homogeneous tubular dentin	Irregular pattern of tubules	Tubular dentin present	Absences

Table 2: Difference between control and MC groups regarding studied items at 1-week duration.

Variable	Score 1		Score2		Score 3		Score 4		P value
	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	
Inflammatory cell extent									
Control	1	16.7	5	83.3	0	0	0	0	0.080
MC	5	83.3	1	16.7	0	0	0	0	
DB formation									
Control	0	0	0	0	6	100	0	0	0.002*
MC	0	0	6	100	0	0	0	0	
Morphology of DB									
Control	0	0	0	0	6	100	0	0	0.002*
MC	0	0	6	100	0	0	0	0	

*Significant result, Fisher's exact test used

Table 3: Difference between control and MC groups regarding studied items at 4-week duration.

Variable	Score 1		Score2		Score 3		Score 4		P value
	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	Frequency N=6	Percent %	
Inflammatory cell extent									
Control	4	66.7	2	33.3	0	0	0	0	0.455
MC	6	100	0	0	0	0	0	0	
DB formation									
Control	0	0	6	100	0	0	0	0	0.002*
MC	6	100	0	0	0	0	0	0	
Morphology of DB									
Control	0	0	6	100	0	0	0	0	0.002*
MC	6	100	0	0	0	0	0	0	

*Significant result, Fisher's exact test use

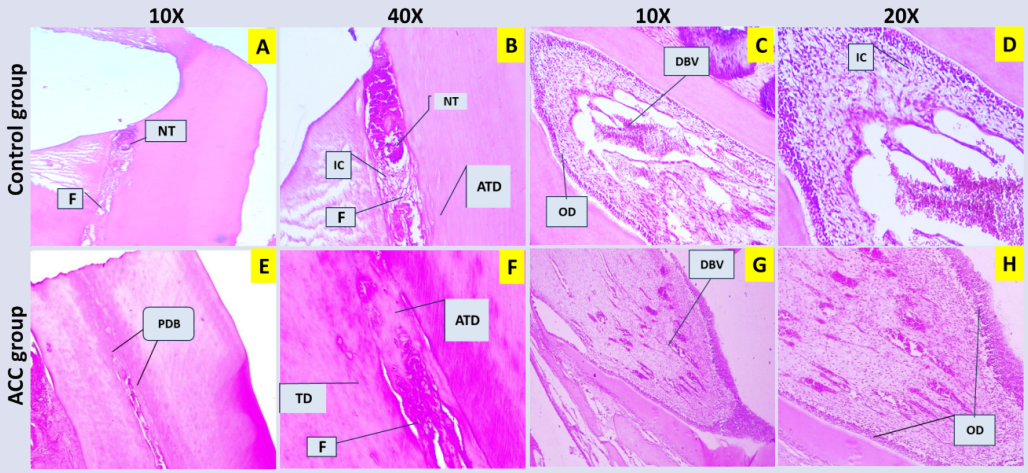

Figure 1: A representative images of the pulp tissues at first week of the control versus ACC group after sectioning and staining with hematoxylin and eosin. NT=necrotic tissue, IC=inflammatory cell, F=fibrous tissue, ATD=A tubular dentin, DBV=Dilated blood vessel, OD=odontoblast, PDB=partial dentin bridge, TD=tubular dentin.

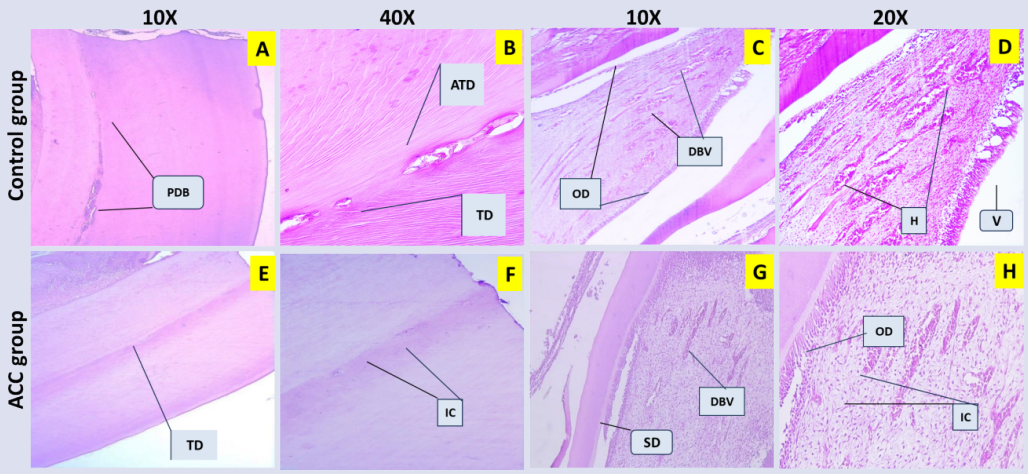

Figure 2: A representative images of the pulp tissues at fourth week of the control versus ACC group after sectioning and staining with hematoxylin and eosin. PDB=partial dentin brigde, OD=Odontoblast, ATD=A tubular dentin, TD=tubular dentin, DBV=dilated blood vessel, V=vacuoles, H=hyalinized blood vessel, IC=Inflammatory cells, SD=secondary dentin, CI=cell inclusion.

However, none of the specimens in both groups showed a severe inflammatory response. Fisher's exact test shows a non-significant difference between the two groups concerning inflammatory response (P=0.08) but there is a highly significant difference in dentin bridge formation and morphology of the formed dentin (P=0.002).

Fourth week histological analysis:

Control group: 66.7% of samples show mild inflammatory response, and 33.3% shows moderate response, with regard to other histologic features of the pulp, diffuse mineralization and hyalinized blood vessel was detected, obvious decrease of the dilated blood vessel compare to 1st week number ,vacuoles also seen in odontoblastic layer, regarding dentin bridge formation and morphology, all samples shows incomplete bridge formation interrupted by void like tunnel defects filled with fibrous tissue and exhibiting mixed pattern of tubular and atubular dentin, corresponding to score two (Table 3, Figures 2A, B, C, and D).

ACC group: all samples showed few dilated blood vessels and few inflammatory cells infiltration, the remaining pulp tissue showed dense fibrous connective tissue, thick complete dentin bridge was formed in most of the specimens, regular secondary dentin were observed in all cases (Table 2, Figures 2 E, F, G, and H).

According to Fisher's exact test, the difference in inflammation grades between the control group and the ACC group was statistically non-significant (p=0.455). Concerning the proportions of the continuity and morphology of dentin bridge formation, there is ahigh significant difference in both parameter(p=0.002), where complete dentin bridge formation was observed in all of the specimens which are mostly tubular dentin.

DISCUSSION

The current study was designed to evaluate the histopathological pulpal response to modified calcite as a potential novel pulpotomy paste using a rabbit's incisor. Rabbit incisor was selected because it represents a valid analogue to the human immature tooth in terms of a large crown size suitable for the restorative procedure, open apex, being free of age changes and due to the histological resemblance of coronal pulp anatomy to that of human being tooth.[36,37] Furthermore, taking into consideration the high proliferative capacity of rabbit incisors with the virtue of the biocompatibility and sealing ability of RMGIC as final restoration rabbit incisors represent a viable candidate for short-term preclinical evaluation.[29,35,38,39] However, continuous tooth growth doesn't pose a problem as the study is short-term histological evaluation rather than long-term clinical evaluation,[36] and the effect of capping material may be monitored up to the apical part of the pulp like in immature human teeth.[29] Furthermore, the histological results obtained from the capping procedure using rabbits incisors are comparable to results obtained from multiple in vitro and in vivo study.[35] In this study, the control group was used to compare auto-repair and material-induced repair.[34] The criteria for histological evaluation included in this study are inflammatory response extent, dentin bridge continuity as well and morphology using Hematoxylin and eosin stains, these features represent the most important histological features for predicting future clinical outcomes according to previous studies.[31,33,34] the evaluation is done in two-time point after 1week to evaluate the initial pulp response and after 4 weeks to evaluate the final tissue response as the rabbit incisors are continuously growing and these period were established by previous studies.[31,40,41] In 1st week period, in the non-capped control group, Although RMGIC cements used to seal the cavity were not in direct contact with the pulp, it's impossible to exclude that they didn't have any effect on the inflammatory process. However, 83.3% of the control group showed moderate pulpal inflammation, which may be due to traumatic exposure procedure,[42] as well as the cytotoxic effect of resin-modified glass ionomer[43]. However, the limited necrotic layer seen underneath the exposure site was neither completely resorbed nor expanded to occupy totally or partially the pulp in any of the examined control specimens at this period, present results agree with the result of using RMGIC in direct contact with the pulp of non-human primate teeth[44,45] and disagree with the histological finding of the control group using rabbit incisors,[32] rat molar[34] as control group, where the pulp shows partial necrosis after 7 days post-capping, it must be noted that both of these studies used the normal saline as irrigant and cotton pressure for hemostasis, unlike to present study. While, as in the present study, the studies that show biocompatibility of RMGIC, using a high concentration of NaOCl for disinfection and to achieve hemostasis preventing blood clot formation, these results refer to the superiority of NaOCl as hemostatic and disinfection irrigant after pulp exposure.[46] Nevertheless, the superficial necrosis may attributed to the limited cytotoxic effect of RMGIC or NaOCl.[46,47] Furthermore, on day 7 control group showed the formation of scanty calcified tissue at the periphery of the exposure site may refer to the high regenerative capacity of rabbit pulp which is not hindered by

the presence of bacteria due to the good sealing ability of RMGIC, these data agree with previous studies that shows the dentin bridge formation can be seen in control and experimental group using rabbit incisors at the day 7 after pulp exposure.[39,40] Meanwhile, in the MC group on day 7, most of the specimens showed mild inflammation and absence of necrotic layer response, indicating the biocompatibility of the MC novel paste, these data greed with the findings of in vivo study where the pure calcium carbonate slurry used to as pulpotomy paste for rat molar which shows mild inflammatory response after 7 days and formation of dentin bridge,[40] also present finding in a line with a recent study which using eggshell slurry as capping material in rabbit incisors, the biocompatibility and bioactivity of eggshell owing to high $CaCO_3$ content which may reach upto 95%by weight.[31] However, the mild inflammatory response to MC may attributed to the mild Alkaline pH of the modified calcite cement due to the release of acetate and bicarbonate group from the paste,[48] which may induce the low-grade inflammatory response of rabbit incisors pulp, this finding agrees with the finding of in vivo study using eggshell paste as capping material to traumatically exposed rabbit incisors[31]. Furthermore, the acetate group has an anti-inflammatory effect and may effectively participate in reducing the initial inflammatory response.[49] Hence, MC material seems to be quite effective in resolving the inflammation earlier, which is essential for rapid dentin bridge formation with good quality.[50] The statistical analysis shows a non-significant difference between groups despite the lower inflammatory response of the MC group These results may explained by the small size of used animal number used, which is not compatible with the number of variables used to determine inflammatory score(inflammatory cell infiltration, dilated blood vessel, disorganization of the tissue and necrosis extent) so we suggest either to increase the number of animal used or use scoring system for each single variable. However, at day 7 there is a highly significant difference between the groups in dentin bridge formation (P=0.002) the MC group shows partial dentin bridge formation with a mixed pattern of dentinal tubule organization, indicating the effectiveness of MC paste in stimulating early dentin deposition, these data agreed with invitro study of culturing hDPCs with calcite, where there is faster deposition of calcified tissue in dose depended mannar,[51] also present data comparable with data of in vivo study using calcium carbonate as pulp capping agent apply on rat molars, calcite group shows early dentin bridge formation compare to calcium hydroxide group owing to biocompatibility of calcite cement and sustained release of Ca^{2+} ion.[52] On day 28, both groups showed mild inflammatory response but there is a highly significant difference in dentin bridge formation where the calcite group shows complete bridge formation of a regular arrangement of dentinal tubules, while the control group shows partially formed dentin bridge of irregular and regular pattern, the healing process of control group proceedings may be attributed to the capacity of the pulp to survive and produce of dentin bridge in absence of microbial leakage,[34,53] as well as the reduction of the possible toxicity of RMGIC cement with time.[54] These findings of the control group partially agree with the four weeks findings of using rabbit incisors,[32] and rat molar as control group,[34] which shows the formation of dentin bridge with the presence of moderate inflammatory persistent response. In the MC group, the pulp tissue shows normal architecture with the absence of diffuse calcification, vacuoles and blood vessel hyalinization compared to the control group which indicates the MC paste is highly biocompatible and doesn't have an adverse effect upon degradation, these findings agree with four weeks findings of the pulp of rabbit incisors after capped with eggshell slurry, where mild inflammatory response and complete dentin bridging was found in 100% of the teeth at 28-day post capping.[31,40] In general, the mild alkaline pH, sustained release of calcium ions and the anti-inflammatory effect of the acetate group seem to support the hypothesis that MC cement is an effective material for capping exposed pulps.

CONCLUSION

MC paste exhibited good biocompatibility with pulpal tissue and induced the early formation of good good-quality reparative dentin bridge. MC cement may be used as a pulp capping material for vital pulp therapy.

CONFLICTS OF INTEREST

The authors have no conflicts of interest relevant to this article.

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